$P2Y_{11}$ receptors: properties, distribution and functions

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Abstract

The $P2Y_{11}$ receptor is a G protein-coupled receptor that is stimulated by endogenous purine nucleotides, particularly ATP. Amongst P2Y receptors it has several unique properties; 1) it is the only human P2Y receptor gene that contains an intron in the coding sequence; 2) the gene does not appear to be present in the rodent genome; 3) it couples to stimulation of both phospholipase C and adenylyl cyclase. Its absence in mice and rats, along with a limited range of selective pharmacological tools, has hampered the development of our knowledge and understanding of its properties and functions. Nonetheless, through a combination of careful use of the available tools, suppression of receptor expression using siRNA and genetic screening for SNPs, possible functions of native $P2Y_{11}$ receptors have been identified in a variety of human cells and tissues. Many are in blood cells involved in inflammatory responses, consistent with extracellular ATP being a damage-associated signalling molecule in the immune system. Thus proposed potential therapeutic applications relate, in the main, to modulation of acute and chronic inflammatory responses.

Index keywords

2-methylthioADP	2-methythioATP	ARC67085MX	ADP
ADPβS	Anti-inflammatory	ATP	ATPαS
ATPγS	Biased agonism	BzATP	cAMP
Canine	Cardiovascular	cytokines	DAMPs
dATP	dendritic cells	epithelia	granulocytes
heteromultimer	inflammation	inositol phosphates	intron
keratinocytes	macrophages	Mesenchymal cells	$NAADP^+$
NAD^+	narcolepsy	Natural killer cells	Neutrophils
NF157	NF340	NF546	*P2RY11*
P2RY11/PPAN	$P2Y_{11}$ receptor	Polymorphisms	SNPs
UDP	UTP	*Xenopus*	

Indexing keywords

$P2Y_{11}$ receptor	ATP	NF157	NF340	NF546

1. Introduction

P2Y receptors are G protein-coupled receptors (GPCR) that are stimulated by the endogenous nucleotides, adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), uridine 5'-triphosphate (UTP), uridine 5'diphosphate (UDP) and UDP-glucose. Eight human subtypes have been cloned ($P2Y_{1,2,4,6,11,12,13,14}$) [1,2] and are expressed throughout the body [3-5]. The missing numbers represent non-mammalian orthologues or proteins proposed to be P2Y receptors, but at which the nucleotides were subsequently found to be inactive. The eight genuine P2Y subtypes show differential sensitivities to the endogenous agonists and the $P2Y_{11}$ receptor is activated by ATP and ADP, but not the uridine nucleotides. In addition, the $P2Y_{11}$ receptor has several unique properties; 1) it is the only human P2Y receptor gene that contains an intron in the coding sequence; 2) the gene does not appear to be present in the rodent genome; 3) it couples to stimulation of both phospholipase C (PLC) and adenylyl cyclase. Here, we will discuss these properties and review the pharmacological profile and possible physiological functions of the $P2Y_{11}$ receptor.

2. Cloning and sequence of $P2Y_{11}$ receptors

Humans The human $P2Y_{11}$ ($hP2Y_{11}$) receptor was first cloned from placenta using probes based on the nucleotide sequence of the $P2Y_4$ receptor [6]. The *P2RY11* gene (AJ298334) is on chromosome 19 (19p13.2) in humans, is 1125 base pairs long and codes a protein of 274 amino acids (CAC29362.1) [7]. Unlike all other P2Y subtypes, the gene has a 1.9 kb intron that separates two exons, which encode the first six amino acids and the rest of the receptor respectively. The $P2Y_{11}$ protein has relatively low sequence identity with the other P2Y subtypes, ranging from 23% ($P2Y_{14}$) to 34% ($P2Y_6$) (Table 1). Multiple single nucleotide polymorphisms (SNPs) have been identified in the human genome and two of these, rs3745601 and rs2305795, will be discussed below when considering possible functions of $P2Y_{11}$ receptors.

Initially, the *P2RY11* receptor sequence was reported to comprise 1113 base pairs (AF030335), coding 371 amino acids (AAB88674.1), but subsequently, the same group reported that this cDNA sequence arose from intergenic splicing of the *P2RY11* gene and that of *PPAN*, the human orthologue of Ssf1, a nuclear protein that is involved in mating in *Saccharomyces cerevisiae*, which lies adjacent and upstream [7,8]. The first *P2RY11* exon was thus revealed to code for the protein sequence MAANVS, rather than MDR, as initially reported. The *P2RY11/PPAN* fusion transcript (AJ300588) lacks the first exon of *P2RY11* and the last two thirds of the final exon in *PPAN* and is of unknown function. It appears to be expressed widely throughout the body and, as will be discussed below, this has been a problem when studying the distribution of *P2RY11* mRNA.

Species orthologues The canine *P2RY11* receptor (NM_001204441) was subsequently cloned [9]. The gene is found in the same synteny as the human gene and the resultant protein has 70% amino acid sequence identity with the $hP2Y_{11}$ receptor. An amphibian p2y receptor (AM040941) that has 35% amino acid sequence identity with the $hP2Y_{11}$ receptor is proposed to be the species homologue of *Xenopus* [10]. Putative orthologues are identified in a number of other species in the NCBI HomoloGene database, but intriguingly, the gene does not appear where expected in the rat and mouse genomes and convincing *P2RY11* mRNA transcripts have not been identified in rodent tissues [2,11,12]. Furthermore, it is unlikely that the gene has translocated to a different chromosomal region and can still be transcribed to produce a functional protein, as using the human sequence to search the UniProt protein database for similar mouse proteins, Dreisig and Kornum, [12] found the closest match to be the $P2Y_1$ receptor, at 32% similarity. This is the same degree of similarity as seen between the human $P2Y_1$ and $P2Y_{11}$ receptors (Table 1). Thus there is no evidence for expression of *P2RY11* mRNA and $P2Y_{11}$ protein in rodents that resemble those present in humans, which must be taken into account when considering pharmacological studies in rodent tissues, as will be discussed below.

3. Coupling of $P2Y_{11}$ receptors to 2nd messenger systems

Direct activation of signalling pathways As P2Y receptors are GPCR, they couple to heterotrimeric G proteins and in this respect they fall into two groups: the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ subtypes couple mainly to $G\alpha_{q/11}$, as indicated by a rise in cytoplasmic levels of inositol 1,4,5-trisphosphate (IP_3) and related inositol phosphates (IPs), whilst the $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors couple to $G\alpha_{i/o}$ [2,13]. The $P2Y_{11}$ receptor is unique in that it couples to both $G\alpha_{q/11}$ and $G\alpha_s$ [14]. When activated, $G\alpha_{q/11}$ stimulates PLCβ, which cleaves phosphatidylinositol 4,5-bisphosphate in the plasma membrane into 1) IP_3, which binds to IP_3 receptors in the endoplasmic reticulum to release Ca^{2+} stores; and 2) diacylglycerol, which activates protein kinase C (PKC). $G\alpha_q$ can also bind to and activate the guanine nucleotide exchange factor, p63RhoGEF, which in turn stimulates Rho GTPases, such as RhoA [15]. $G\alpha_s$, on the other hand, activates adenylyl cyclase, leading to increased synthesis of cAMP and subsequently stimulation of protein kinase A.

Initially, ATP was reported to activate adenylyl cyclase with similar [6] or greater potency [14] compared to PLC activation. The experiments were, however, carried out in different cell lines; 1321N1 human astrocytoma cells for IP accumulation and CHO-K1 cells for cAMP synthesis. Since agonist potency is greatly influenced by the level of receptor expression [16,17], the coupling of the $hP2Y_{11}$ receptor to these signalling pathways was studied further in the same cell line [18]. In

1321N1 cells, ATP promoted IP accumulation with low μM potency (EC_{50} = 8.5 ± 0.1 μM) and was 15-fold less potent in raising cAMP (EC_{50} =130 ± 10 μM). In CHO-K1 cells, ATP evoked IP accumulation with slightly higher potency (EC_{50} = 3.6 ± 1.3 μM) than in 1321N1 cells, but it was still 15-fold less potent in promoting cAMP synthesis (EC_{50} = 62.4 ± 15.6 μM). Comparable differences in potencies for promoting the two second messenger responses were observed with other adenosine nucleotide analogues. Thus the $P2Y_{11}$ receptor appears to couple more effectively with the $G\alpha_{q/11}$ signalling pathway than that of $G\alpha_s$.

Indirect activation of adenylyl cyclase It is important to note that some isoforms of adenylyl cyclase can be activated by PKC, independently of $G\alpha_s$ [19]. For example, $P2Y_1$ receptors can activate PKCζ, which increases the activity of isoform 5 of adenylyl cyclase [20]. Such a mechanism does not appear to underlie the rise in cAMP mediated by $P2Y_{11}$ receptors, as inhibition of PKC did not supress cAMP production elicited by stimulation of the canine $P2Y_{11}$ ($cP2Y_{11}$) receptor stably expressed in CF2Th cells [9]. Concomitant activation of PKC may, however, potentiate cAMP synthesis, as down-regulating PKC by chronic treatment with a phorbol ester decreased ATP-promoted cAMP accumulation by 60-80%, with no change in ATP's potency, in both 1321N1 and CHO-K1 cells expressing the $hP2Y_{11}$ receptor [18]. Likewise, chelation of intracellular Ca^{2+} decreased ATP-promoted cAMP accumulation by ~45% in 1321N1 cells, whereas chelation had no effect on either the efficacy or potency of ATP in CHO-K1 cells. Thus the capacity of the $P2Y_{11}$ receptor to elicit cAMP accumulation appears to be via $G\alpha_s$, and this can be potentiated by the concomitant activation of PKC and mobilisation of intracellular Ca^{2+} stores in a cell-type- and/or species subtype-dependent manner.

4. Pharmacological properties of $P2Y_{11}$ receptors

Agonists The $hP2Y_{11}$ receptor is adenine nucleotide-preferring, with ATP more potent than ADP, whilst UTP, UDP and the nucleotide triphosphates, guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate, thymidine 5'-triphosphate and inosine 5'-triphosphate (ITP), are inactive [6,18,21]. The rank order of agonist potency for increasing either cAMP or IP_s is ARC67085MX ≥ ATPγS ≈ BzATP > dATP > ATP ≈ 2-methythioATP ≈ ATPαS > ADPβS > 2-methylthioADP > ADP [6,14,18,22,23,24]. In addition, the diphosphates, ADP, ADPβS and 2-methylthioADP are partial agonists, with apparent efficacies of 60-80% of maximal response to ATP [23]. Note that AR-C67085XX, the most potent $P2Y_{11}$ agonist, also antagonises $P2Y_{12}$ receptors [13], which limits its usefulness in characterising the functions of native $P2Y_{11}$ receptors. These studies all used the

original $hP2Y_{11}$ clone, in which the N terminal amino acid sequence begins MDR. When the receptor with the correct sequence, MAANVS, was expressed, there was, however, no significant difference in the potency of ATP and related molecules between the two isoforms [24].

Screening of a library of sulphonic and phosphonic acid derivatives identified NF546, a full agonist at $hP2Y_{11}$ receptors [24]. It is 2.5-fold less potent than ATP, but has a degree of selectivity over other P2Y subtypes as its EC_{50} value at $hP2Y_{11}$ receptors was 28-fold, 102-fold and 604-fold lower than that at human $P2Y_2$, $P2Y_6$ and $P2Y_{12}$ receptors respectively and it had little or no effect at human $P2Y_1$ and $P2Y_4$ receptors. Its activity at other P2X and P2Y subtypes has not been reported. Apparent agonist potency in bioassays depends, however, on the level of receptor expression, so whilst NF546 is a very useful tool for the pharmacological characterisation of native P2Y receptor subtypes, an action over a particular concentration range is not necessarily evidence for the presence of $P2Y_{11}$ receptors and an appropriate antagonist action profile and/or knockdown of the receptor using siRNA must also be obtained.

Nicotinamide adenine dinucleotide (NAD^+) and nicotinic acid adenine dinucleotide phosphate ($NAADP^+$), which both play important roles in intracellular signalling, are also $hP2Y_{11}$ agonists [25,26]. Furthermore, it has been suggested that NAD^+ can be released from cells via connexin 43 hemichannels and so may act endogenously [27]. Note, however, that NAD^+ also stimulates other P2 subtypes, including P2X1, P2X4, P2X7 and $P2Y_1$ receptors [28,29]. In addition, NAD^+ can be metabolised to cyclic ADP-ribose, a potent agonist for release of intracellular Ca^{2+} stores [30], so a pharmacological effect of NAD^+ cannot, on its own, be automatically taken as evidence for the expression of $P2Y_{11}$ receptors and again and an appropriate antagonist action profile and/or knockdown of the receptor using siRNA must also be obtained.

Antagonists Suramin and PPADS are general, non-selective P2X and P2Y antagonists [5,31] and whilst suramin, at low μM concentrations, is a surmountable $hP2Y_{11}$ antagonist, PPADS is inactive [14,22]. The suramin analogue, NF157, is a competitive antagonist with $pA_2 = 7.77$, which makes it more potent than suramin, and it is at least 650-fold selective over $P2Y_1$ and $P2Y_2$ receptors [32]. In addition, NF157 is non-selective over P2X1 receptors and has low (P2X2, P2X3) to moderate (P2X4, P2X7) selectivity over other P2X subtypes [32]. Its activity at other P2X and P2Y subtypes has not been reported. These factors can limit its usefulness when studying native $P2Y_{11}$ receptors and care must be taken when using NF157 to obtain supporting data, such as lack of effect of a range of other P2X and P2Y antagonists and/or depression of receptor expression using tools such as siRNA.

Another suramin analogue, NF340, is also a competitive antagonist, with a pA_2 of 8.02 [24].

10 μM NF340 inhibited responses at recombinant human $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors by less than 10% and so NF340 was calculated to have at least 520-fold selectivity over other P2Y receptors. Similarly, stimulation of recombinant human P2X1, P2X2 and P2X2/3 receptors was unaffected by 3 μM NF340. This compound may, therefore be more useful in determining the physiological functions of native $P2Y_{11}$ receptors, but again, its activity at other P2X and P2Y subtypes has not been reported and supporting data should be obtained.

UTP is not a biased agonist at $P2Y_{11}$ receptors Biased agonism describes a multi-state model of GPCR activation in which each ligand induces a unique structural conformation of the receptor, such that the receptor couples differentially to G proteins and other intracellular proteins. For example, activation of the $P2Y_2$ receptor by UTP causes similar translocation of β-arrestin-1 and 2 from the cytoplasm to the plasma membrane, whereas ATP induced a greater translocation of β-arrestin-1 [33]. These differential effects of ATP and UTP had a downstream effect on cell signalling, as both agonists increased the levels of phosphorylated extracellular-signal regulated kinase, but the effect of UTP was transient, whereas that of ATP was prolonged.

As discussed above, all of the initial studies found UTP to be ineffective at raising IPs or cAMP in cells expressing $hP2Y_{11}$ receptors. A subsequent report proposed, however, that UTP was a biased $P2Y_{11}$ agonist, as it increased cytosolic [Ca^{2+}], but did not induce accumulation of IPs, whereas ATP did both [34]. This was an intriguing proposal, so we studied the action of UTP at $P2Y_{11}$ receptors in greater detail [21].

We found that ATP evoked a rapid, concentration-dependent rise in intracellular Ca^{2+} with an EC_{50} that was essentially the same as that reported by [34]. This is not surprising, as the 1312N1-$hP2Y_{11}$ cell line used in both studies was generated by the present author whilst on sabbatical at the University of North Carolina. Despite this, we did not observe a rise in intracellular Ca^{2+} when UTP was applied, even at 100 μM, a concentration that was almost maximally effective in the earlier study (Figure 1). Consistent with this, another group also failed to see a UTP-induced rise in intracellular Ca^{2+} levels in 1321N1 cells stably expressing the $hP2Y_{11}$ receptor [24]. Furthermore, we found that coapplying a high concentration of UTP with ATP did not inhibit either the rise in Ca^{2+} or IPs evoked by ATP (Figure 2), indicating that UTP does not bind to this receptor.

In contrast, UTP was slightly, but significantly more potent than ATP in evoking a rise in intracellular Ca^{2+} in 1321N1 cells stably expressing the human $P2Y_2$ receptor, with no difference in the maximum response. Thus the lack of response to UTP at $hP2Y_{11}$ receptors was not due to a problem with the UTP solution. So, contrary to the previous report, we found no evidence for an agonist action of UTP at the $hP2Y_{11}$ receptor, nor does UTP act as an antagonist. In our view the

most feasible explanation for the discrepancy in the activity of UTP is contamination of the UTP solution by a non-nucleotide agent acting at a receptor that is not a P2Y receptor in the earlier study.

cP2Y$_{11}$ pharmacology Interestingly, the cP2Y$_{11}$ receptor is adenine diphosphate-preferring, with reported rank order of agonist potencies of ADPβS = 2-methylthioADP ≈ 2-methylthioATP >> ADP > ATP [9] and 2-methylthioADP > ADPβS > 2-methylthioATP > ADP > ATPγS ≥ ATP [23] for both IP and cAMP production. Furthermore, ADP, ADPβS and 2-methylthioADP are full agonists [23]. Like the hP2Y$_{11}$ receptor, UTP, UDP, GTP and ITP were inactive, and suramin, but not PPADS inhibited these responses.

The human and canine receptors have only 70% amino acid identity and a mutagenesis study targeted the arginine residue (Arg268) that is present at the junction between TM6 and the third extracellular loop of the hP2Y$_{11}$ receptor, by replacing it with glutamine, which is present in the analogous position in the cP2Y$_{11}$ receptor [23]. This change increased both the potency and efficacy of ADP relative to ATP, such that they were now nearly equipotent and equi-efficacious. Likewise, mutating the glutamine in the canine receptor to arginine increased the efficacy and potency of ATP relative to ADP, and they now had essentially identical potency and efficacy. Thus this single amino acid within the P2Y$_{11}$ receptor is at least partially responsible for the species differences in its pharmacological properties.

Ligand binding site Computational modelling and mutational analysis have provided insight to some of the amino acid residues that likely play a role in ligand binding. As noted above, Arg268 in the hP2Y$_{11}$ receptor is at least partially responsible for the differences in the relative potency of ATP and ADP compared with at the canine orthologue [23]. This amino acid also plays a major role in agonist stereo-selectivity. The hP2Y$_{11}$ receptor is preferentially activated by the Rp stereoisomer of ATPαS, but mutating Arg268 to an uncharged residue caused the stereo-selectivity to be lost [35]. This mutational approach has also implicated arginine 106 and 307, glutamate 168 and alanine 313 in the ATP binding pocket [36]. Determination the three dimensional structure of the P2Y$_{11}$ receptor through crystallisation or similar high resolution techniques is required, however, to confirm this.

5. P2Y$_1$/P2Y$_{11}$ heteromultimers

GPCR were long considered to be monomeric entities that couple to G proteins on a 1:1 stoichiometric basis. It is now clear, however, that GPCR, including P2Y receptors, can interact to

form dimeric or higher ordered oligomeric complexes, which may couple to one or more G proteins, with implications for the regulation of receptor subcellular localisation and trafficking, ligand binding and functional activity [37-40]. Constitutive formation of $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ homodimers has been shown using biochemical techniques [41,42,43] and publication of the crystal structure of the $P2Y_{12}$ receptor confirmed the latter [44]. Furthermore, different P2Y subtypes can interact physically to form function heterodimers, with altered pharmacological and signalling properties (see, for example, [45,46,47].

This is true also for the $P2Y_{11}$ receptor, which forms a complex with $P2Y_1$ receptor that has a greatly altered sensitivity to the antagonists, MRS2179 and NF157 [48]. In addition, the $P2Y_{11}$ receptor did not undergo agonist-induced endocytosis when expressed on its own, consistent with the early report that $P2Y_{11}$ receptors do not desensitise [14]. However, after cotransfection of the $P2Y_1$ subtype, ATP induced endocytosis of the $P2Y_{11}$ protein, which was not inhibited by the $P2Y_{11}$ antagonist NF157 [48]. The authors, therefore, concluded that agonist-induced endocytosis of the $P2Y_{11}$ receptor requires coexpression of the $P2Y_1$ receptor and in a subsequent study identified alanine 87 of the $P2Y_{11}$ receptor as playing a crucial role in the interaction with $P2Y_1$ receptors [49]. In addition, G protein receptor kinase 2 may also be required for $P2Y_{11}$ internalisation [33].

6. Expression of $P2Y_{11}$receptors

mRNA expression Many studies using RT-PCR or northern blotting have reported detection of *P2RY11* mRNA in a range of tissues and species [2], but this is complicated by the discovery that the *P2RY11*/*PPAN* fusion mRNA transcript is expressed widely (see [12] for an extensive overview). The latter transcript comprises the second exon of *P2RY11* and most of the sequence of *PPAN*, so RT-PCR and northern blotting will only distinguish between the two sequences if one of the primers is designed to recognise at least part of the first exon of *P2RY11* and/or part of the untranslated upstream sequence.

After a detailed and extensive review of the published literature, Dreisig and Kornum [12] identified only 5 papers out of 89 in which detection of the fusion transcript could confidently be discounted, all in human cells [50-54]. Thus it is clear that care must be taken when designing primers and that many reports that claimed to show *P2RY11* mRNA expression need to be revisited and reviewed. The same group subsequently reported detection of *P2RY11* mRNA in distinct regions of macaque monkey brain [55].

Protein expression The presence of mRNA may not necessarily mean that the corresponding protein is expressed, so it is essential to confirm protein expression using a selective antibody. This

approach has been used widely to study expression of all P2Y receptor subtypes, but in most cases the specificity of the antibody has not been unequivocally confirmed, for example, by showing that staining is absent in the appropriate receptor knock-out animal. Indeed, a commonly-used anti-$P2Y_1$ antibody has been reported to be nonspecific [56] and we found that several commercially-available P2Y subtype antibodies show staining in 1321N1 cells, which do not express endogenous P2Y receptors (Kennedy, unpublished observations). Thus care must be taken when interpreting antibody staining data.

This holds true for data obtained using anti-$P2Y_{11}$ antibodies, as a review of the published literature identified several issues with the commercially-available anti-$P2Y_{11}$ antibodies [12]. Most of these target a region of the C-terminus of the receptor that has significant sequence similarity with the C-terminus of other P2Y subtypes, so there is a reasonable chance that this antibody may not be specific for the $P2Y_{11}$ receptor. Although the predicted size of the $P2Y_{11}$ protein is 40 kDa, western blot data from different groups, in which the most commonly used antibody (#APR-015) was utilised, reported the molecular mass to be 33-60 kDa. All claimed that their band represented the monomeric $P2Y_{11}$ receptor (see [12] for full details).

Anti-$P2Y_{11}$ antibodies also bind proteins in rodent tissues [2,12], even though the *P2RY11* gene is not present in the rodent genome, further casting doubt upon their specificity. An attempt to verify the specificity of three commercially-available anti-$P2Y_{11}$ antibodies was made by first determining if they recognised the recombinant $hP2Y_{11}$ receptor when expressed in a cell line [55]. One antibody that targeted the 3rd cytoplasmic loop of the $hP2Y_{11}$ receptor did not show any signal of the expected band size in western blots, but instead produced two bands of 142 and 195 kDa, including in untransfected cells. The other two antibodies, targeted against the 3rd extracellular loop or the C-terminus, both produced multiple bands, including of the expected size. These two antibodies stained neuronal-like cells in macaque monkey brain, but an identical staining pattern was also seen in rat brain. Thus the specificity of these antibodies could not be validated.

Finally, the targeted C-terminus region is also present in the $P2Y_{11}$/PPAN fusion protein, which has a predicted size of 90 kDa, so that too will be detected by anti-$P2Y_{11}$ antibodies. For example, an antibody generated against a sequence at the end of the $P2Y_{11}$ C-terminus produced a band of around 90 kDa in western blots using CHO-K1 cells transfected with the *P2RY11/PPAN* fusion plasmid, whereas transfection with the *P2RY11* plasmid resulted in bands of around 45 kDa [7]. Transfection with the empty vector produced no bands.

7. Functions of $P2Y_{11}$receptors

Criteria for identifying receptor function Ideally, a comprehensive understanding of the

function of any receptor arises from characterisation of the effects of highly selective agonists and antagonists on cellular and tissue activity and correlation of these with the expression of its mRNA and protein and perhaps also with the effects of deleting the gene or supressing translation of the mRNA. Unfortunately, since the *P2RY11* gene appears to be absent in mice and rats, the species used in genetic deletion studies, it is not possible to produce $P2Y_{11}$-knock-out animals. In addition, as discussed above, doubt has been cast on the specificity of commercially-available anti-$P2Y_{11}$ antibodies. Nonetheless, *possible* functions of native $P2Y_{11}$ receptors have been identified in a range of non-rodent cells by applying two or more of the following criteria: a) detection of mRNA using appropriately designed primers; b) demonstration of appropriate effects of appropriate concentrations of NF546, NF157 and NF340; c) lack of effect of agonists and antagonists selective at other P2Y and P2X subtypes; d) inhibition of the proposed $P2Y_{11}$ function by *P2RY11* RNA interference. Detection of a band close to the predicted size of the $P2Y_{11}$ receptor, ~40 kDa, in western blots, although not direct evidence in itself, might also be considered consistent with $P2Y_{11}$ expression in a tissue.

Mostly, the proposed functions are based on single reports, though they may be supported by other studies in the same cell type in which only one of the above criteria are fulfilled. The majority of studies used siRNA knock-down of $hP2Y_{11}$ receptors to support pharmacological data and many are in blood cells involved in immune responses, consistent with extracellular ATP being a member of the damage-associated molecular patterns (DAMPs) family [57].

Granulocytes β-NAD^+ elicited rises in cytoplasmic Ca^{2+}, IP_3 and cAMP in freshly isolated human granulocytes, which were inhibited by 1 μM NF157, (a concentration that is 50-times higher than the antagonist's affinity for $hP2Y_{11}$ receptors) and downregulation of $P2Y_{11}$ expression by siRNA [25]. Furthermore, NF157 inhibited β-NAD^+-induced granulocyte chemotaxisand it was suggested that β-NAD^+ is an endogenous $hP2Y_{11}$ agonist and acts as a proinflammatory cytokine. Subsequently, similar effects were seen with another endogenous $P2Y_{11}$ agonist, $NAADP^+$ [26].

Neutrophils Human neutrophils undergo constitutive apoptosis, which was delayed by ATP, NAD^+ and BzATP, but not UTP and appeared to be dependent upon cAMP activation of PKA [58], consistent with earlier reports of a $P2Y_{11}$-mediated increase in cAMP in human HL-60 cells, which are comprised mainly of neutrophil promyelocyte precursor cells [59,60]. The delay in apoptosis was inhibited by 500 nM NF157, but unaffected by P2X7 antagonism. Western blotting identified a protein of approximately 45 kDa. Similar results were reported in [61], which further demonstrated that the delay in neutrophil apoptosis was due to inhibition of the mitochondrial, but not the

extrinsic, pathway of apoptosis. Thus endogenous ATP, and possibly also NAD^+, may act via $P2Y_{11}$ receptors to increase neutrophil survival, which will prolong the inflammatory response and the ability of neutrophils to phagocytose and destroy foreign particles and invading microorganisms. Further supporting evidence is, however, required.

Macrophages THP-1 cells are a human acute monocytic leukemia cell line that differentiate into the M1-proinflammatory type of macrophage after priming with lipopolysaccharide (LPS) and interferon-γ. This was inhibited by apyrase, a soluble enzyme that dephosphorylates ATP, and by 50 μM NF157 [62]. This is a high concentration of NF157 (2,500-times greater than its K_B at $P2Y_{11}$ receptors) and is enough to produce substantial blockade of numerous P2X and P2Y subtypes. THP-1 differentiation, was, however, unaffected by a range of other antagonists, PPADS (non-selective P2X and P2Y antagonist), NF449 (P2X1 antagonist), A438079 (P2X7 antagonist), MRS2179 ($P2Y_1$ antagonist), MRS2578 ($P2Y_6$ antagonist), clopidogrel ($P2Y_{12}$ antagonist), MRS2211 ($P2Y_{13}$ antagonist), consistent with NF157 acting here via $P2Y_{11}$ receptors. Furthermore, THP-1 differentiation was also supressed by down-regulation of $P2Y_{11}$ receptors by specific siRNA.

LPS also induced the release of the pro-inflammatory cytokine, IL-6, and this too was inhibited by NF157 and $P2Y_{11}$-specific siRNA. Inhibition of adenylyl cyclase, similarly, depressed IL-6 release, consistent with IL-6 release being mediated by $P2Y_{11}$ receptors. Based on these and other results, the authors concluded that LPS induces vesicular exocytosis of ATP from macrophage precursor cells, which then acts in an autocrine manner at $P2Y_{11}$ receptors to cause these cells to differentiate into M1-pro-inflammatory macrophages that release pro-inflammatory cytokines, such as IL-6. In addition, they proposed that $P2Y_{11}$ receptor antagonists might potentially be useful in the treatment of inflammatory diseases, such as sepsis.

Dendritic cells The first clear evidence for functional expression of $P2Y_{11}$ receptors in human dendritic cells was that the $P2Y_{11}$ agonist, NF546, induced a rise in intracellular Ca^{2+} concentration that was abolished by NF340 [24], which is consistent with expression of *P2RY11* mRNA in these cells [51]. In contrast to the studies above, which indicate pro-inflammatory roles for $P2Y_{11}$ receptors, an anti-inflammatory role is indicated in human cultured, monocyte-derived dendritic cells [63]. Here, ATP and BzATP, but not ADP, UTP or UDP, induced dendritic cell maturation, which was inhibited by 10 μM NF340 and suramin, but unaffected by PPADS and P2X4 and P2X7 antagonists. In these cells, LPS caused release of the inflammatory cytokine, IL-12 and this was inhibited by ATP, an action that was reversed by both NF340 and down-regulation of $P2Y_{11}$

receptors by specific siRNA. Exposing dendritic cells to hypoxia for 5 hours, followed by reoxygenation, also suppressed the inhibitory effects of ATP and in addition, down-regulated *P2RY11* mRNA. The authors proposed that this inhibition of $P2Y_{11}$ receptor activity may cause dendritic cells to become pro-inflammatory and so contribute to post ischaemia/reperfusion injury.

Bronchial epithelial cells The human bronchial epithelial cell lines, NuLi-1 and CuFi-1, are amongst the small number of cell types in which expression of *P2RY11* mRNA has been demonstrated with confidence [53]. This group further showed that in these cells, lipoxin A4 (LXA_4), a regulator of adaptive immunity that has been proposed to reduce inflammation, acts by inducing ATP release, which in turn stimulates $P2Y_{11}$ receptors. In these cells, LXA_4 raised cytoplasmic Ca^{2+} and cAMP, increased airway surface liquid height, induced cell proliferation and migration and improved wound repair, all of which were inhibited by NF340 (0.1-1 μM). In addition, knock-down of the $P2Y_{11}$ receptor using siRNA inhibited the increase in airway surface liquid height. Thus in this situation it is stimulation, rather than inhibition, of $P2Y_{11}$ receptors that is likely to be beneficial therapeutically.

Keratinocytes $P2Y_{11}$ receptor-mediated release of pro-inflammatory cytokines is also indicated in keratinocytes [64]. Interferon-γ induced IL-6 release from the human HaCaT keratinocyte cell line and this was inhibited by apyrase, suramin and NF157. The concentration of NF157 used, 100 μM, was very high, but IL-6 release was unaffected by a range of other antagonists; PPADS (non-selective P2X and P2Y antagonist), AZ10606120 (P2X7 antagonist), MRS2179 ($P2Y_1$ antagonist), MRS2578 ($P2Y_6$ antagonist), clopidogrel ($P2Y_{12}$ antagonist), MRS2211 ($P2Y_{13}$ antagonist). Furthermore, IL-6 release was also inhibited by down-regulation of $P2Y_{11}$ receptors using specific siRNA. Thus $P2Y_{11}$ receptors may play a role in inflammatory conditions of the skin, such as psoriasis and blocking them could facilitate skin repair.

Mesenchymal cells NAD^+ evoked rises in cytoplasmic Ca^{2+}, cAMP and cyclic ADP-ribose in human bone marrow-derived mesenchymal stem cells, which were all abolished by 1 μM NF157 [27]. Although alternative antagonists were not employed to rule out the involvement of other P2X and P2Y receptor subtypes, the effects of NAD^+ were also inhibited by $P2Y_{11}$ receptor down-regulation by siRNA, consistent with NAD^+ acting here via $P2Y_{11}$ receptors. In the same study, NAD^+ also induced NF157-sensitive chemotaxis of the mesenchymal cells, a small increase in cell proliferation, release of a variety of cytokines and increased nuclear translocation of a number of

cAMP/Ca^{2+}-dependent transcription factors. The cells also released NAD^+ and ATP via connexin43 hemi-channels, suggesting that these nucleotides may act as endogenous modulators of mesenchymal cell function.

8. *P2RY11* polymorphisms as an indicator of function Numerous SNPs of the *P2RY11* gene are identified in the NCBI Single Nucleotide Polymorphism database and whilst their functional consequences are mostly unknown, genetic screening has implicated two SNPs in disease states.

Cardiovascular disease Genotyping of participants in the Malmo Diet and Cancer (MDC) study identified a small increased risk of acute myocardial infarction (AMI) in the A87T polymorphism (rs3745601) and the risk was greater in patients with a family history of AMI and/or early onset AMI [65]. In addition, patients in the MDC cardiovascular risk group had elevated plasma levels of C-reactive protein, a marker of inflammation and a strong prognostic factor for the development of AMI. Thus this $P2Y_{11}$ polymorph appears to be associated with increased systemic inflammation and risk of cardiovascular disease.

Position 87 is near the extracellular end of the second transmembrane spanning region of the $P2Y_{11}$ receptor and the $P2Y_{11}$A87T variant replaces a hydrophobic alanine residue with a polar threonine. How this could lead to the changes seen in the MDC cohort is unclear, but the A87T mutation had no effect on the potency and efficacy of BzATP compared with wild-type $P2Y_{11}$ receptors expressed on their own, but BzATP became inactive when h$P2Y_{11}$A87T was coexpressed with $P2Y_1$ receptors [49]. This was not mimicked by replacing A87 with serine or tyrosine. Additionally, the A87T mutation rendered the receptor insensitive to agonist-induced internalisation and the authors concluded that it disrupts the $P2Y_1$/$P2Y_{11}$ heteromeric receptor interaction.

Narcolepsy Increasing evidence indicates that narcolepsy is an autoimmune disease that leads to a loss of hypocretin (orexin)-releasing neurones in the hypothalamus. Genome-wide association studies have identified a SNP (rs2305795) that is associated with narcolepsy in European and Asian [51], Chinese [66] and Japanese [67] patients. The SNP is in a region of the 3′ untranslated sequence of *P2RY11* that appears to regulate transcription, as it correlated with substantially reduced expression of *P2RY11* mRNA in $CD8^+$ T lymphocytes and natural killer cells of the carriers, which in turn was associated with a reduced ability of $P2Y_{11}$ receptors to inhibit ATP-induced monocyte cell death [51]. These data are consistent with roles for $P2Y_{11}$ receptors in modulation of immune cell function, as discussed above.

9. $P2Y_{11}$-like receptors in rodents As discussed in section 2, the *P2RY11* gene has not been identified in rodent genomes and careful analysis has failed to reveal good evidence for expression of *P2RY11* mRNA and $P2Y_{11}$ protein in rodents that resemble those present in humans. Nonetheless, there is a body of reports that propose functional $P2Y_{11}$ receptor expression in rodent tissues. Some are based on the ability of ATP to raise cytosolic cAMP, but, as discussed in section 3, some isoforms of adenylyl cyclase can be activated independently of $G\alpha_s$ and so an increase in cAMP cannot, on its own, be taken as evidence for $P2Y_{11}$ expression. In the main, the reports are based on pharmacological data using agonists, such as NAD^+, and/or NF157, as a supposedly selective $P2Y_{11}$ antagonist. As discussed in section 4, however, both compounds have multiple sites of action and should not be described as "selective". Furthermore, no report has convincingly excluded these other sites from the effects of NAD^+ or NF157 by using a variety of subtype-selective antagonists. In addition, data obtained using NF340 or NF546, which are more selective that NAD^+ and NF157, cannot be supported by knock-down of the receptor by siRNA due to the lack of the *P2RY11* gene. Consequently, until a solution to the apparent lack of *P2RY11* gene in rodent genomes has been found, these receptors should, at best, only be termed $P2Y_{11}$-like.

10. Concluding remarks In the 20 years since the $P2Y_{11}$ receptor was first cloned, our knowledge and understanding of its properties and functions have improved relatively slowly. This has been, in part, due to its apparent absence in mice and rats, the most commonly used species for studying receptors and drug action and which prevent genetic deletion studies from being performed. In common with many other purinergic receptor subtypes, progress has also been hampered by the limited range of selective pharmacological tools. Nonetheless, by careful use of the available tools, along with suppression of receptor expression using siRNA and also genetic screening for SNPs, possible functions of native $P2Y_{11}$ receptors have been identified in a range of human cells and tissues. Many are in blood cells involved in inflammatory responses, consistent with extracellular ATP being a DAMP signalling molecule in the immune system. Thus proposed potential therapeutic applications relate, in the main, to modulation of acute and chronic inflammatory responses. Further study of these in animals will require the development of non-rodent models, whilst selective ligands with suitable pharmacokinetic properties are needed before potential trials in humans can go ahead. Regardless of these difficulties it is clear that the $P2Y_{11}$ receptor is a viable, novel therapeutic target.

Conflicts of interest

The author declares that he has no conflict of interests.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Abbreviations

ADP - adenosine 5'-diphosphate

AMI - acute myocardial infarction

ATP - adenosine 5'-triphosphate

cP2Y$_{11}$ receptor - canine P2Y$_{11}$ receptor

DAMPs - damage-associated molecular patterns

GPCR - G protein-coupled receptors

GTP - guanosine 5'-triphosphate

hP2Y$_{11}$ receptor - human P2Y$_{11}$ receptor

IP$_3$ - inositol 1,4,5-trisphosphate

IPs - inositol phosphates

ITP - inosine 5'-triphosphate

LXA$_4$ - lipoxin A$_4$

MDC - Malmo Diet and Cancer

NAADP$^+$ - nicotinic acid adenine dinucleotide phosphate

NAD$^+$ - nicotinamide adenine dinucleotide () and

PKC - protein kinase C

PLC - phospholipase C

SNPs - single nucleotide polymorphisms

UDP - uridine 5'diphosphate

UTP - uridine 5'-triphosphate

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	% identity							
Subtype	$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	$P2Y_{11}$	$P2Y_{12}$	$P2Y_{13}$	$P2Y_{14}$
$P2Y_1$	-	38	44	46	32	24	24	27
$P2Y_2$		-	41	41	29	25	26	26
$P2Y_4$			-	43	32	25	26	28
$P2Y_6$				-	34	24	24	23
$P2Y_{11}$					-	22	21	23
$P2Y_{12}$						-	48	48
$P2Y_{13}$							-	47
$P2Y_{14}$								-

Table 1. Human P2Y receptor subtype amino acid sequence identity

Figure Legends

Figure 1. ATP, but not UTP increases intracellular Ca^{2+} in 1321N1-hP2Y_{11} cells.

a) The superimposed traces show fluo-4 fluorescence during superfusion with ATP (10 μM) (upper trace) and UTP (100 μM) (lower trace) for 90 sec, as indicated by the horizontal bar. Both records are from the same population of cells. b) The mean peak amplitude of responses evoked by ATP (n=5) and UTP (10 μM, n= 6 and 100 μM, n=12) are shown. Responses are expressed as % of the response to ATP (10 μM). Vertical lines show S.E.M. From Morrow et al., (2014).

Figure 2. UTP does not inhibit ATP-evoked responses in 1321N1-hP2Y_{11} cells.

a) The traces show the rise in intracellular Ca^{2+} evoked by ATP (2 μM) in the same population of cells before (left-hand side) and after superfusion for 10 min with UTP (100 μM) (right-hand side), as indicated by the horizontal bars. b) The mean peak amplitude of responses evoked by ATP (2 μM) in the absence and presence of UTP (100 μM, n= 5) are shown. Responses are expressed as % of the control response to ATP (2 μM). c) The mean basal level of IPs (left-hand column) and the mean amplitude of responses evoked by ATP (3 μM) in the absence (middle column) and presence of UTP (100 μM) (right-hand column) are shown. n=3. Vertical lines indicate S.E.M. From Morrow et al., (2014).